Long Fiber Formation of Hydroxyapatite/Collagen Nanocomposites through a Self-Organization Mechanism

M. Kikuchi*, J. Tanaka, Biomaterials Center, NIMS, Tsukuba, Ibaraki, Japan;
S. Itoh, K. Shinomiya, Orthopedic Surgery, Tokyo Med. & Dent. Univ., Bunkyo-ku, Tokyo, Japan

Abstract

Long fibers of bone-like hydroxyapatite/collagen nanocomposites were fabricated via a self-organization under biomimetic conditions in order to obtain much more suitable biomechanical and biochemical properties for artificial bone and tissue engineering scaffolds. The fiber growth was controlled with ionic strength in the reaction vessel and was explained by a crystal growth model. The composite fiber grew up to 75 mm in length and demonstrated straight extinction under a polarized microscope. The composite compacts were incorporated into bone metabolism, and their resorption was controlled with crosslinkage. The composite is utilizable for the bone reconstruction materials that can gradually change into bone.

Introduction

Bone is a well-designed nanocomposite functioned as vertebrates' structural materials and calcium reservoir [1]. In bone, collagen forms periodic bundles, along which hydroxyapatite nanocrystals are aligned, resulting in good mechanical properties. The nanostructure of bone also could be closely related to its metabolism because without this kind of nanostructure, the materials cannot substitute with bone tissue via bone remodeling process even the materials are composed of hydroxyapatite (HAp) and collagen [2, 3].

Since Bioglass® had been developed by Hench, bioceramics has been being developed worldwide to substitute structural function of bone for bone defects caused by injuries and deceases. In fact, bioceramics usually indicates good biocompatibility to living tissue in comparison to biotolerant metal artificial bones; bioinert ceramics, *e.g.*, alumina and zirconia, demonstrate high mechanical strength and no inflammations and bioactive ceramics, *e.g.*, Bioglass®, hydroxyapatite and A-W glass ceramics, reveal direct bone bonding properties. Although hydroxyapatite lacks high mechanical properties, it has been used in medical and dental fields as bone fillers and coating materials on metal due to its similarity of composition to bone minerals as well as bioactivity. However, these materials usually remain for a long time even they indicate a possibility of resorption by osteoclasts *in vitro* and gradual dissolution by chemical reactions. The remaining of biomaterials in bone usually caused serious problems such as bone fracture after bone absorption or stress shielding around the materials; therefore, bioresorbable artificial bone materials are requested from physicians to manage patients' quality of life. The first approach to bioresorbable artificial bone is to use biodegradable materials, *e.g.*, polylactide based polymers [4], highly soluble ceramics such as β-tricalcium phosphate [5], calcium carbonate [6] and calcium sulfate [7], and their composites [8, 9]. Although some of them demonstrated adequate in biodegradable properties and osteoconductivity, they still have a problem that they cannot cooperate with cells, especially osteoclasts and osteoblasts that play important roles in a bone remodeling process, because they generally degrade chemically but biologically by osteoclasts. The second one is to use biopolymers, mainly collagen sponges. The biopolymers actually decomposed by a biological process; however, they are not sufficient for artificial bone because they resorbed by a phagocytosis of macrophages and have no mechanical strength, usually collapse with swelling by adsorption of aqueous solution. The third and most sophisticated way is the use of organic and inorganic bone components, collagen and HAp, with similar nanostructure to bone; however, as mentioned above, it is very difficult to mimic nanostructure of bone and they are not showing obvious evidence that the materials incorporate into bone remodeling process.

The authors assumed that the nanostructure of bone was self-organized via chemical interaction between surface ions on HAp and functional groups on collagen because this structure is too small to fabricate with tentacles of cells, and have been reported the reproduction of bone-like nanostructure by use of simple apparatus (Fig. 1) [10]. Further, the bone-like HAp/Col nanocomposites are resorbed by osteoclasts followed by osteogenesis by osteoblasts, *i.e.*, incorporated into bone remodeling process [10-14]. In the present paper, HAp/Col composite fibers of 75 mm in length were synthesized by controlling a self-organization condition closely to control mechanical properties and micro- and macro-structure. The biological reaction of the resultant composite was examined by animal tests.

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 JUN 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Long Fiber Formation of Hydroxyapatite/Collagen Nanocomposites through a Self-Organization Mechanism	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Biomaterials Center, NIMS, Tsukuba, Ibaraki, Japan; Orthopedic Surgery, Tokyo Med. & Dent. Univ., Bunkyo-ku, Tokyo, Japan**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001697, ARO-44924.1-EG-CF, International Conference on Intelligent Materials (5th) (Smart Systems & Nanotechnology)., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**6**	

Materials and Method

Hydroxyapatite/collagen (HAp/Col) composite fibers were synthesized via self-organization process using $Ca(OH)_2$, H_3PO_4 (Reagent grade, Wako Pure Chemicals Ind.) and type-I atelocollagen (Biomaterial Grade, Nitta Gelatin Inc.) as starting materials. Calcium hydroxide was prepared by hydration of CaO, which was obtained by decarbonation of $CaCO_3$ (Alkaline analysis grade, Wako Pure Chemicals Ind.) at 1050 °C for 3 h to avoid existence of trace Mg. Type-I atelocollagen was prepared by pepsin treatment of purified type-I collagen extracted from porcine dermis. The concentration and amount of the starting materials (Table 1) were determined as total mass of the composite to be 10 g after ideal reaction of $Ca(OH)_2$, H_3PO_4 and the atelocollagen. Schematic drawing of synthesize apparatus is shown in Fig. 1. Distilled deionized water was previously added in a reaction vessel to measure pH of reaction solution from a starting point of synthesis; the amount was the same as $Ca(OH)_2$ suspension. The HAp/Col composite was synthesized at pH 9 and 40 °C by a simultaneous titration method [10] at a titration rate of 15 cm^3/min of $Ca(OH)_2$ and H_3PO_4 solutions. Calcium ion concentration in the reaction solution was measured by Ca ion electrode (DKK-TOA Co.). Hydroxyapatite was synthesized by the same method using 800 cm^3 of 100 mM $Ca(OH)_2$ and 1600 cm^3 of 30 mM H_3PO_4 as a reference of an oscillation of Ca ion concentration. The length of HAp/Col fibers synthesized was measured with a Rapid-VUE® system (Beckman Coulter Inc.) up to 1 mm and with a ruler over 1 mm. The fibers were also picked up with forceps one by one, observed with a polarized microscope and analyzed by an X-ray diffractometry. The composite was further filtrated and formed into a cylindrical shape by dehydration using uniaxial pressing. The composite compact was cut into 5×3×20 mm^3; 3-point bending strength was measured by a universal testing machine (AGS-1kN, Shimadzu) at a crosshead speed of 500 μm/min with a span of 15 mm. The inorganic/organic ratios of the composite fibers were measured with thermogravimeter-differential thermal analyzer (TG-DTA, Thermoplus, Rigaku Co.) Animal test was carried out by veterinarian in accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication # 85-23 Rev. 1985). The composite compacts were implanted into bone holes on tibia of Wistar rats.

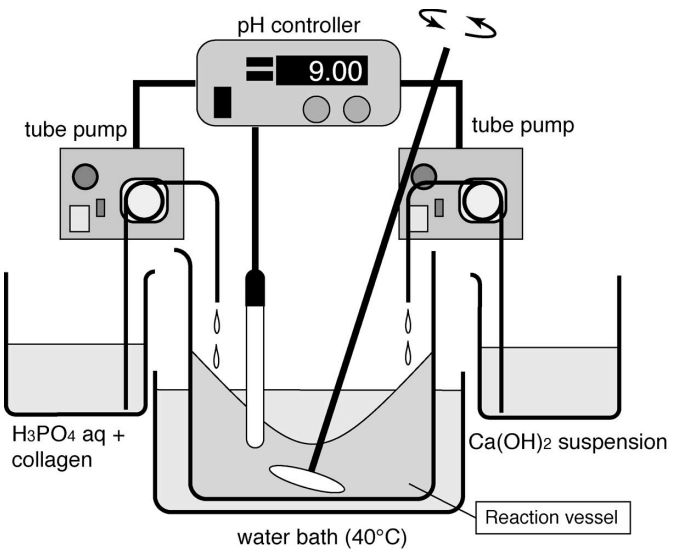

Fig. 1. Schematic drawing of synthesis apparatus for the hydroxyapatite/collagen composite fibers. (After Kikuchi *et.al*.[10])

Table 1. Concentration and amount of starting materials.

$Ca(OH)_2$ suspension	Conc. / mM	50	100	200	300	400	400
	Amount / cm^3	1600	800	400	166.7	200	200
H_3PO_4 solution	Conc. / mM	15	30	60	90	120	120
	Amount / cm^3	3200	1600	800	333.3	400	400
Collagen	2	4					
HAp/Col mass ratio		80/20					60/40

Results and Discussion

As shown in Fig.2, the HAp/Col fibers grew longer and thicker with decreasing in concentration of the reaction solutions. The maximal length of the fibers was about 20 mm at a calcium concentration of 100 mM, while it is 20 μm or less at a higher concentration [10]. From X-ray diffraction, the inorganic phase of the composite fibers prepared at a target HAp/Col mass ratio of 80/20 was identified as a hydroxyapaite single phase and they at 60/40 were identified as hydroxyapatite and a small amount of $CaCO_3$. Calcium carbonate is considered as remains of starting materials that could not react with PO_4 sufficiently. The mass ratios of the composite fibers prepared at a target HAp/Col mass ratio of 80/20 were in the range of 76/24 to 81/19 and they at 60/40 were 57/43 to 61/39. The mass ratio of collagen seemed to tend to increase with decreasing in calcium starting concentration and increasing in fiber length but not obviously. The results suggest that the reaction among $Ca(OH)_2$, H_3PO_4 and atelocollagen almost ideally progressed. Polarizing microscopic photographs of the

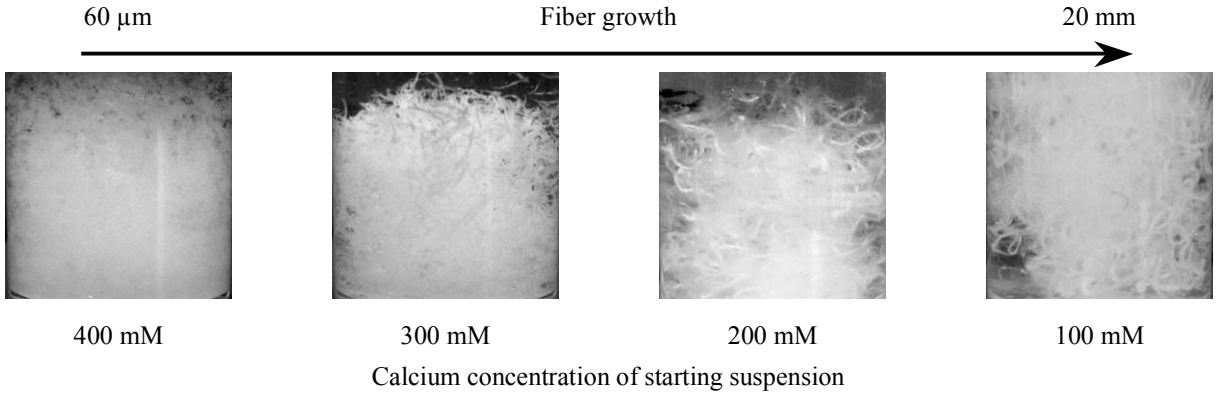

Figure 2. HAp/Col self-organized fibers prepared at various concentrations

composite fiber prepared at a target HAp/Col mass ratio of 60/40, shown in Fig. 3, demonstrated straight extinction of fiber, *i.e.*, orientation of collagen microfibrils.

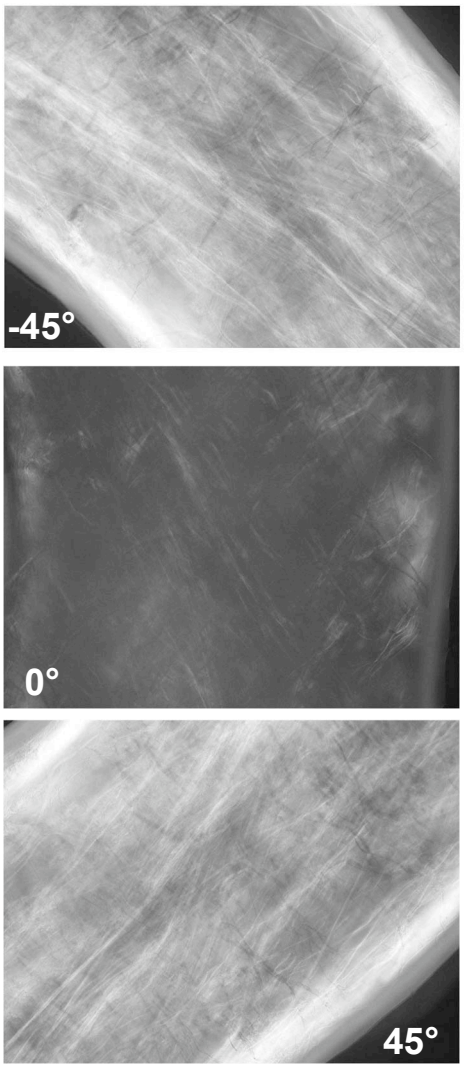

Figure 3. Polarizing microscopic photographs of HAp/Col composite fiber. HAp/Col mass ratio is 60/40.

In Fig. 4, the time course of Ca ion concentration in the reaction vessel is indicated. The Ca ion concentrations of a starting suspension and a reaction solution were correlative and each calcium concentration of all reaction solutions was higher enough than that of HAp saturated solution but about a quarter of that of human blood plasma in which HAp does not precipitate. The variation of calcium concentrations for the HAp synthesis and for the calcium concentration of 200 mM in the starting suspension were greater than that for the other conditions, and mean concentration was also relatively high. This difference was explained by the nucleation of hydroxyapatite crystal and the fibrillogenesis of collagen molecules. Without collagen, the homogeneous nucleation of hydroxyapatite occurred only from a supersaturated solution. Generally, hydroxyapatite crystals could not grow larger even in this supersaturated condition because of its low solubility and complicated growth system in comparison to other inorganic crystals [15]. As a result, the next homogeneous nucleation must occur at higher concentration than equilibrium one for hydroxyapatite crystals and calcium, phosphate and hydroxyl ions to reach the equilibrium. Consequently, the calcium concentration in the reaction vessel was unstable and varied widely. Under the existence of collagen, however, hydroxyapatite crystals can form on carboxyl groups of collagen that act as nucleation centers to decrease the activation energy [10,16]. In addition, the hydroxyapatite formation on the carboxyl groups promoted the fibrillogenesis of collagen molecules though Ca ions were generally inhibitors for the fibrillogenesis [17]. As a result, heterogeneous hydroxyapatite formation on collagen molecules and collagen fibrillogenesis highly activated by hydroxyapaite on collagen occurred simultaneously and continuously at low concentration of starting materials. Although collagen was added in the reaction solution, in the case of higher starting calcium concentration than 200 mM, addition of high concentration of starting materials led both homogeneous in solution and heterogeneous on collagen functional group nucleation of hydroxyapatite crystals due to acute raise of calcium (and phosphate) concentrations. Coinstantaneously, collagen amounts in the reaction vessel became larger and possibility of heterogeneous nucleation of hydroxyapatite on collagen became higher. This condition led a preparation of large amounts of short fibers of the HAp/Col composite and they cannot join each other to grow as long fibers [collagen]. Because of these complex conditions, fiber length at calcium starting concentration of 200 mM (and also 300 mM) widely changed from 1 mm to 20 mm in each synthesis. Further, these conditions in the reaction vessel at the calcium starting concentration higher than 400 mM (data not shown) could not

allow growing long composite fibers due to high mean ion concentration in the vessel. These mechanisms explain that the fiber growth of the HAp/Col composite behaves as the same as the crystal growth from supersaturated solution. Although collagen fibril growth is usually described as the similar process as the crystal growth, collagen fibrils cannot grow longer from physiological saline solution of collagen. *i.e.*, collagen concentration does not depend on fibril length [collagen]. The results suggested that the presence of hydroxyapaite enhanced fiber growth of collagen molecules.

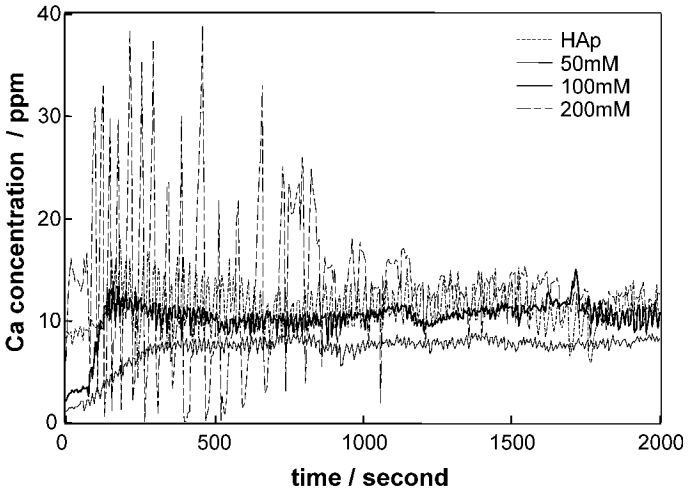

Figure 4. Time course of calcium concentration in the reaction vessel.

The fiber growth led higher 3-point bending strength to the composite about 20 MPa in comparison to 10 MPa with short fibers as shown in Fig. 5. At the calcium starting concentration of 200 mM, fiber lengths were very different in each experiments because of the reason mentioned above. Therefore, bending strength was significantly different for each synthesis as seen in wide standard deviation of bending strength. From these results, reinforcement effect on the compact was increased with fiber length. However, at a calcium starting concentration of 50 mM, even the self-organized fibers grown up to 30 mm in maximum length, the bending strength and Young's modulus decreased drastically. With increasing in fiber length, water content of the compact increased in particularly 50 mM of calcium concentration (55 mass% instead of 25-30 mass% in the other conditions). The major reason why is the fibers packed into the mold to dehydration could not be compacted dense by an elasticity of fibers and sizes. The elasticity of fibers caused re-hydration of the surrounding removed water in dehydration apparatus to the compact after pressing. The large size of fibers obviously inhibits dense packing as the same as to preparing green body for sintering. The minor one is the tendency to contain larger amounts of collagen in the fibers compared to other conditions because collagen can hold larger amounts of water than hydroxyapaite. Water content is also important for biomaterials because it closely relates to affinity to water, *i.e.*, absorbing properties of enzymes and cytokines and attachment of cells. Therefore, control of fiber length is very important to prepare artificial bone and scaffold materials having suitable mechanical and biochemical properties.

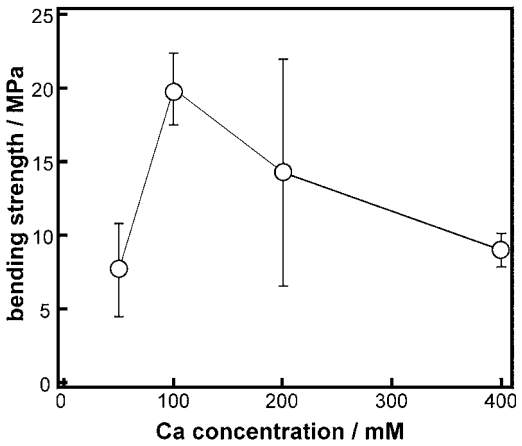

Figure 5. Changes in bending strength of HAp/Col composite as a function of starting Ca concentration.

The results of animal tests demonstrated that the composite was incorporated into a bone remodeling process, *i.e.*, the composite was resorbed by osteoclastic cells followed be new bone formation by osteoblasts in the lacunae on the composites. The coupling of the osteoclastic resorption and osteogenesis continued for at least 4 weeks. This obvious remodeling process, quite similar to that observed for autogenous bone transplantation, has never been observed before the HAp/Col self-organized nanocomposites *in vivo* [10], though many results in osteoclasis of hydroxyapatite ceramics [18] and □-tricalcium phosphate ceramics [19] *in vitro* are reported. Some reports say the ceramic and composite materials resorbed by osteoclasts *in vivo* [2, 3, 20], they demonstrates a few or no evidence in their paper concerning the formation of the bone remodeling unit. The present study demonstrates that the remodeling process *in vivo* occurred with not only bone-like composition of materials but self-organized bone-like nanostructure even the composite fiber size grow to centimeter scale. Subsequently, cells can detect the nanostructure of materials through their nano-specific physicochemical properties.

Conclusion

The HAp/Col self-organized naocomposite fibers are grown by a control of both hydroxyapatite formation and collagen fibrillogenesis via management of starting materials concentration. The maximum length of the composite fiber was grown to 75 mm at a Ca starting concentration of 100 mM and HAp/Col mass ratio of 60/40. The HAp/Col composite fibers can

be utilize for scaffold of bone tissue engineering due to their shape to easily prepare sponge, net and sheet as well as their osteoactivity that incorporated into bone remodeling process.

References

1. Neuman WF, Neuman MW, "The chemical dynamics of bone mineral", University of Chicago Press, Chicago, 1958.

2. Mehlisch DR, Leider AS, Roberts WE. Histologic evaluation of the bone/graft interface after mandibular augmentation with hydroxylapatite / purified fibrillar collagen composite implants. Oral Surg Oral Med Oral Pathol 1990; 70(6): 685-692.

3. Du C, Cui FZ, Zhu XD, de Groot K, Three-dimensional nano-HAp/collagen matrix loading with osteogenic cells in organ culture., *J Biomed Mater Res*, 1999; 44: 407-415

4. Hasegawa Y, Sakano S, Iwase T, Warashina H. The long-term behavior of poly-L-lactide screws in a minipig fracture model: Preliminary report. J Biomed Mater Res 2002;63(6):679-85.

5. Ogose A, Hotta T, Hatano H, Kawashima H, Tokunaga K, Endo N, Umezu H. Histological examination of β-tricalcium phosphate graft in human femur. J Biomed Mater Res. 2002; 63(5):601-4.

6. Damien CJ, Ricci JL, Christel P, Alexander H, Patat JL. Formation of a calcium phosphate-rich layer on absorbable calcium carbonate bone graft substitutes. Calcif Tissue Int. 1994; 55(2): 151-8.

7. Kelly CM, Wilkins RM, Gitelis S, Hartjen C, Watson JT, Kim PT. The use of a surgical grade calcium sulfate as a bone graft substitute: results of a multicenter trial. Clin Orthop. 2001; 382: 42-50.

8. Sikinami Y, Okuno M. Bioresorbable devices made of forged composites of hydroxyapatite particles and poly-L-lactide (PLLA): Part I. Basic characteristics. *Biomaterials*, 1999; 20:859-877.

9. Kikuchi M, Koyama Y, Takakuda K, Miyairi H, Shirahama N, Tanaka J. *In vitro* change in mechanical strength of β-tricalcium phosphate/copolymerized poly-L-lactide composites and their application for guided bone regeneration. J Biomed Mater Res. 2002; 62(2): 265-72.

10. Kikuchi M, Itoh S, Ichinose S, Shinomiya K, Tanaka J. Self-organization mechanism in a bone-like hydroxyapatite/collagen nanocomposite synthesized *in vitro* and its biological reaction *in vivo*. Biomaterials. 2001; 22(13): 1705-11.

11. Itoh S, Kikuchi M, Takakuda K, Koyama Y, Matsumoto HN, Ichinose S, Tanaka J, Kawauchi T, Shinomiya K. The biocompatibility and osteoconductive activity of a novel hydroxyapatite/collagen composite biomaterial, and its function as a carrier of rhBMP-2. J Biomed Mater Res. 2001; 54(3): 445-53.

12. Itoh S, Kikuchi M, Koyama Y, Takakuda K, Shinomiya K, Tanaka J. Development of an artificial vertebral body using a novel biomaterial, hydroxyapatite/collagen composite. Biomaterials. 2002; 23(19): 3919-26.

13. Itoh S, Kikuchi M, Takakuda K, Nagaoka K, Koyama Y, Tanaka J, Shinomiya K. Implantation study of a novel hydroxyapatite/collagen (HAp/Col) composite into weight-bearing sites of dogs. J Biomed Mater Res. 2002;63(5):507-15.

14. M. Kikuchi, T. Taguchi, H. N. Matsumoto, K. Takakuda, J. Tanaka. Key Eng. Mater. 2002: 218-220: 449-52.

15. Kanzaki N, Treboux G, Onuma K, Tsutsumi S, Ito A. Calcium phosphate clusters. Biomaterials. 2001; 22(21): 2921-9.

16. Sato K, Kumagai Y, Tanaka J. Apatite formation on organic monolayers in simulated body environment. J Biomed Mater Res. 2000; 50(1): 16-20.

17. T. Hayashi: "Collagen fibrillogenesis" in Fundament of Bioscience 3 (The Biophysical Society of Japan ed.) (Japan Scientific Societies Press, Tokyo 1977) (in Japanese).

18. Gomi K, Lowenberg B, Shapiro G, Davies JE. Links Resorption of sintered synthetic hydroxyapatite by osteoclasts in vitro. Biomaterials. 1993; 14(2): 91-6.

19. Zheng Q, Du J, Xia Z, Zeng H, Li S, Yan Y, Chen F. Biodegradation of tricalcium phosphate ceramics by osteoclasts. J Tongji Med Univ. 1998; 18(4): 257-61.

20. Takikawa K, Akao M. Fabrication of transparent hydroxyapatite and application to bone marrow derived cell/hydroxyapatite interaction observation *in vivo*. J Mater Sci: Mater in Med 1996; 7: 439-445.